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14. ABSTRACT Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in communication (verbal and nonverbal), social interactions, and stereotyped behaviors/interests. The etiology of ASD is not well understood, though it likely involves both genetic and environmental factors. Immune system dysfunction has been reported in ASD in many studies. Systemic immunologic alterations in autistic individuals often have been associated with autoimmunity; in particular, the generation of antibodies reactive against brain and CNS proteins. The goal of this grant is to identify serum antibody biomarkers for ASD using a novel combinatorial peptoid library that has been successful for the identification of antibody biomarkers for Alzheimer's disease. An ASD blood biomarker would be very useful for early identification and targeted therapeutic intervention. During Year-3 of the grant we have: (1) collected serum samples from additional male typically developing (TD) and ASD subjects; (2) tested the optimal peptoid for sensitivity and specificity using 74 ASD boys, 60 TD boys and for comparison 53 adult males; and (3) we have a research publication in press describing our autism blood biomarker. In addition, we have identified two new proteins that are linked to ASD.					
15. SUBJECT TERMS ASD, autism spectrum disorders. TD, typically developing control. US, unaffected sibling control.					
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Introduction: Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in communication (verbal and nonverbal), social interactions, and stereotyped behaviors/interests. The etiology of ASD is not well understood, though it likely involves both genetic and environmental factors. Immune system dysfunction has been reported in ASD subjects and in their mothers in many studies (e.g., Ashwood & Van deWater, 2004; Jyonouchi *et al.*, 2005; Molloy *et al.*, 2006; Braunschweig *et al.*, 2013). Systemic immunologic alterations in autistic individuals often have been associated with autoimmunity; in particular, the generation of antibodies reactive against brain and CNS proteins. For example, both abnormalities in serum antibody concentrations and T cells have been reported for ASD compared to typically developing (TD) children (e.g., Warren *et al.*, 1990; Singh, 2009). The goal of this study is to identify a serum antibody biomarker for ASD using a novel combinatorial peptoid library that has been successful for the identification of antibody biomarkers for Alzheimer's disease (Reddy *et al.*, 2011). An ASD blood biomarker would be very useful for early identification and targeted therapeutic intervention.

Overall Project Summary:

For Year 3 we proposed to:

1. Determine the Sensitivity and Specificity of the optimal peptoid for the identification of ASD.

We have collected a total of 74 male ASD, 60 male TD, and for comparison purposes 53 adult male (AM) control subjects (mean age – 65 years). We found that the ASD1 peptoid binds ~50% less IgG1 antibody in ASD boys vs. TD boys ($p=0.0096$). The level of ASD1 binding to the AM group was the same as to the ASD boys. These data suggest that the immune system of the ASD boy is similar to that of the older individual, vs. to the TD boy. We know the immune system is weaker as we age and it appears that the immune system of the ASD boy is like that of the older male (see **Figure 1**). The *Sensitivity* of the ASD1 peptoid for identifying ASD vs. TD is 78% and the *Specificity* is 52%. The area under the ROC curve = 0.630 ($p=0.0097$). We found that the binding level of the ASD1 peptoid is negatively correlated with the level of Communication measured on the ADI-R test ($p=0.023$) consistent with lower levels of ASD1 binding going with higher levels of communication problems among the ASD boys.

2. Identify the antibody recognized by the ASD1 peptoid.

We have pulled down the antibody/antibodies that bind to the ASD1 peptoid and run the protein on an electrophoresis gel (see **Figure 2**). We found a single band of approximately 56 kD on the gel, and greater amount pulled down from the ASD pooled serum vs. TD pooled serum. Future study will be required to identify the antibody/antibodies recognized by the peptoid.

Additional efforts

Rules Based Medicine biomarker discovery

In addition to the peptoid approach to identifying biomarkers for ASD, we have used a second approach. Using the Rules Based Medicine DiscoveryMAP 175+ luminex platform, we measured 175 serum proteins in the blood of 28 ASD boys and 28 age-matched TD boys. **Table 1** below shows the 11 proteins that were significantly different between the two groups, and also shows the “importance” of each protein as members of a panel to successfully predict ASD vs. TD after analysis with Random Forest methods. Highlighted in yellow are 6 proteins that we propose to further test with a larger sample of ASD and TD subjects *for validation purposes*, and using a different platform. We have done this with TSH, which has the highest importance for predicting ASD vs. TD. Using the Meso Scale Discovery electrochemical detection platform we found significantly lower TSH in the ASD boys vs. TD boys ($n=50/\text{group}$, $p<0.001$).

Key Research Accomplishments:

- Nearly all serum samples have been obtained and processed.
- Two unique peptoid libraries have been synthesized and validated.
- The peptoid libraries have been screened using a magnetic screening method, and peptoids were found that demonstrate a difference in IgG-binding activity between ASD, TD and AM sera.
- A peptoid, ASD1, has been identified that binds significantly lower levels of IgG1 in ASD boys vs. TD boys.
- Pull-down assays were run to identify the antibody recognized by the ASD1 peptoid.
- Efforts are underway to identify the antibody that binds to the ASD1 peptoid.
- Two new proteins were identified with the ASD1 peptoid with possible links to ASD (TSH and IL8). These proteins may be useful as part of a panel of proteins that can serve as a biomarker for ASD. TSH and IL8 levels were 82% accurate in predicting ASD vs. TD in boys.

Conclusions:

Several peptoids that differentiate between ASD and TD serum have been identified following screenings of a peptoid library. Contrary to expectation, however, these peptoids bind *lower* levels of IgG in ASD serum compared to TD serum. The ASD1 peptoid binds significantly lower levels of IgG1 in ASD boys (n=74) compared to TD boys (n=60). The peptoid was 66% accurate in predicting ASD vs. TD. When levels of ASD1 were combined with serum TSH levels from the same boys the predictive accuracy increased to 73%. These data suggest that a panel of biomarkers may be useful for the identification of ASD.

Reportable Outcomes:

Yazdani, U., Zaman, S., Gadad, B., Li, W., Roatch, N., Schutte, C., Hewitson, L., German, D.C. Blood biomarkers for autism: Peptoids. *Neuroscience Abstract*, 585.05, 2015.

Zaman S, Yazdani U, Deng Y, Li W, Gadad B, Karp D, Roatch N, Schutte C, Hewitson L, German DC. A search for blood biomarkers for autism: peptoids. *Scientific Reports*, 2015, in press.

References:

- Ashwood P, Van deWater J. (2004) Is autism an autoimmune disease? *Autoimmun Rev*, 3:557–62.
- Braunschweig D, Krakowiak P, Duncanson P, Boyce R, *et al.* (2013) Autism-specific maternal autoantibodies recognize critical proteins in developing brain. *Transl Psychiatry*.3: e277.
- Elsas J, Sellhaus B, Herrmann M, Kinkeldey A, Weis J, Jahnen-Dechent W, Häusler M. (2013), Fetuin-A in the developing brain. *Devel Neurobio*, 73: 354–369.
- Jyonouchi H, Geng L, Ruby A, Zimmerman-Bier B. (2005) Dysregulated innate immune responses in young children with autism spectrum disorders: their relationship to gastrointestinal symptoms and dietary intervention. *Neuropsychobiol* 51:77–85.
- Molloy CA, Morrow AL, Meinzen-Derr J, Schleifer K, *et al.* (2006) Elevated cytokine levels in children with autism spectrum disorder. *J Neuroimmunol* 172:198–205.
- Reddy MM, Wilson R, Wilson J, Connell S, Gocke A, Hynan L, German D, Kodadek T. (2011) Identification of candidate IgG antibody biomarkers for Alzheimer's disease through screening of synthetic combinatorial libraries. *Cell*, 144: 132-142.
- Russo, AJ, Neville L, Wroge C. (2009) Low serum alpha-1 antitrypsin (AAT) in family members of individuals with autism correlates with PiMZ genotype. *Biomarker Insights*, 4: 45-56.
- Singh VK. (2009) Phenotypic expression of autoimmune autistic disorder (AAD): A major subset of autism. *Ann Clin Psychiat*. 21:148-160.

Thevenon J, Callier P, Poquet H, Bache I, et al. (2014) 3q27.3 microdeletional syndrome: a recognizable clinical entity associating dysmorphic features, marfanoid habitus, intellectual disability and psychosis with mood disorder. *J. Med. Genet.*, 51:21-27.

Warren RP, Yonk LJ, Burger RA, Cole P, et al. (1990) Deficiency of suppressor-inducer (CD4⁺CD45RA⁺) T cells in autism. *Immunol Invest* 19:245–51.

Appendices:

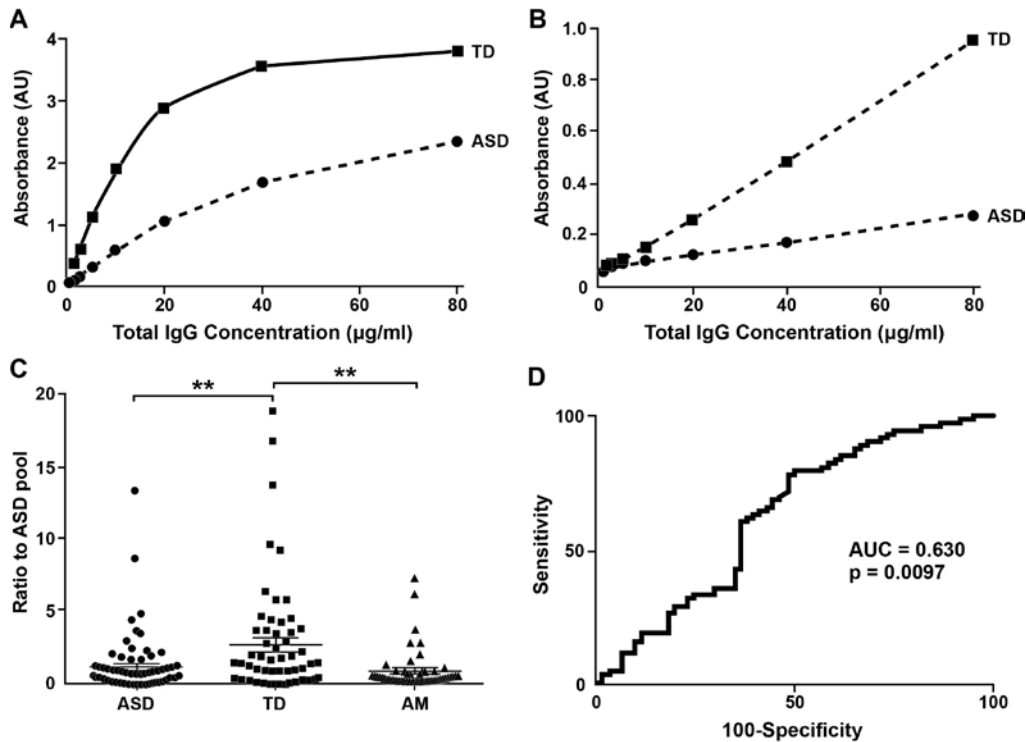


Figure 1. Serum IgG binding to the ASD1 peptoid. (A) Titration of IgG binding to ASD1 using serum pooled from 10 TD males and 10 ASD males demonstrates ASD1's ability to differentiate between the two groups. (B) Detecting IgG1 subclass instead of total IgG amplifies this differentiation. (C) IgG1 binding of individual ASD (n=74) and TD (n=60) male serum samples (1:100 dilution) to ASD1 significantly differs with TD>ASD. In addition, IgG1 binding of older adult male (AM) serum samples (n=53) to ASD1 is significantly lower than TD males, and not different from ASD males. The three groups were compared with a Kruskal-Wallis ANOVA, $H=10.1781$, $p<0.006$. ** $p<0.005$. Error bars show SEM. (D) Receiver-operating characteristic curve for ASD1's ability to discriminate between ASD and TD males.

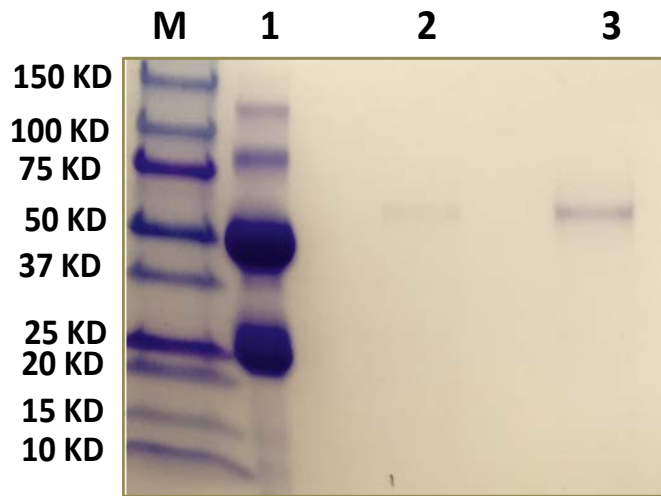


Figure 2. Assessment of proteins that bind to ASD1. The ASD1 peptoid was immobilized and incubated with pooled serum from ASD or TD males. Serum was removed and what proteins were left bound to ASD1 were eluted out and evaluated by gel electrophoresis and Coomassie Blue staining. Lane 1 shows ASD1 pull-down analytes from the ASD serum pool and Lane 2 shows the pull-down from the TD serum pool. Both show a single band at ~55 – 60 kD that is higher in intensity for the TD male analyte.

Table 1. Proteins that differ in ASD vs. TD serum samples (n=28/group). Serum protein measurements from the RBM Luminex platform. A panel of 11 serum proteins were combined to predict ASD among ASD and TD boys (n=28/group). Using random forest analysis, the *importance* for each protein in predicting ASD vs. TD is illustrated. The predictive accuracy of this panel of 11 serum proteins was >65%.

	Protein names	Change	t-test	Importance
1	Alpha 1 Microglobulin - A1Micro	9%↑	0.017	2.309
2	Apolipoprotein E – ApoE	22%↑	0.035	-1.865
3	Apolipoprotein H – ApoH	15%↑	0.103	4.153
4	AXL Receptor Tyrosine Kinase – AXL	11%↑	0.059	-0.233
5	Chromogranin A – CgA	21%↑	0.050	-0.418
6	Ferritin – FRTN	29%↑	0.056	3.295
7	Interleukin 8 – IL-8	31%↑	0.043	2.568
8	Monocyte Chemotactic Protein 4 - MCP4	18%↑	0.064	3.393
9	Monokine Induced by Gamma Interferon – MIG	26%↑	0.166	2.088
10	Stem Cell Factor – SCF	16%↑	0.008	4.356
11	Thyroid Stimulating Hormone – TSH	31%↓	0.003	14.639